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Research Article

IN VITRO & IN VIVO ANTIOXIDANT AND HEPATOPROTECTIVE POTENTIAL OF *CARALLUMA ADSCENDENS* VAR. *ATTENUATA* AGAINST ETHANOL TOXICITY

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Abstract:

Caralluma adscendens var. *attenuata* is a succulent plant grows in tropical areas. It has been reported for the presence of phenolics, flavanoids, tannins etc., which are responsible for antioxidant activity and hepatoprotection. Several biological activities like anti diabetic, anti-inflammatory and anti-ulcerogenic were also reported. The present study was aimed to evaluate invitro antioxidant property as the prime step to carryout invivo antioxidant and hepatoprotective activity of methanolic stem extract of this plant. In vitro antioxidant activity was evaluated by determination of total phenolics, flavonoids content, reductive ability and free radical scavenging activity assays. In vivo studies were performed against ethanol induced liver toxicity in rats using Silymarin as standard. In this study serum catalase, superoxide dismutase, reduced glutathione levels were quantified. The results obtained by this study revealed that the concentration of the liver protecting enzymes was not affected by ethanol when animals were coadministered with plant extract. Rats which were pretreated with methanolic extract of *Caralluma adscendens* var. *attenuata* inhibited the stimulated serum levels of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP) and total bilirubin when compared with silymarin. The research findings reveal that *Caralluma adscendens* var. *Attenuata* has significant antioxidant and hepatoprotective activities.

Keywords: Alcoholism; Hepatotoxicity; *Caralluma adscendens* var. *Attenuata*; Antioxidants; silymarin; Hepatoprotection.

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1. INTRODUCTION:

The liver is known as the chemical factory of the human body which regulates the metabolism of food, xenobiotics and drugs. Healthy habits commonly protect and regenerate hepatic cells if any damage or injury to the liver occurs accidentally, environmental factors or even by the consumption of the drugs. But chronic alcoholism causes severe damage to the liver cells which may develop hepatitis, alcoholic steato necrosis because of excessive production of cytokines (TNF-alpha), Interleukins (IL6 and IL8) [1]. During metabolism alcohol is converted to acetaldehyde, a highly toxic substance and also it liberates acetate anions. If these anions react with hydrogen atoms of the body generates highly reactive free radicals causing Reactive oxidative stress (ROS). Acute free radical accumulation depletes the equilibrium between ROS production and ROS removal [2]. The enzymes cytochrome P450 2E1 (CYP2E1) and catalase becomes active and plays an important role in conversion of alcohol to acetaldehyde. Small amounts of alcohol are removed by interacting with fatty acids to form reactive compounds called fatty acid ethyl esters (FAEEs). However chronic accumulation of these reactive species certainly contributes to hepatic tissue damage & hepatitis [3].

Many edible plants and herbs are widely distributed in nature and found to possess hepatoprotective activity as they contain essential chemical constituents such as phenolics and flavanoids [4]. The literature review supported that *Caralluma adscendens* var. *Attenuata* was chewed by tribals to treat peptic ulcers and wounds. It was also reported for phenolics, flavonoids, tannins, terpenes and alkaloids as chief chemical constituents. In the present study the researcher has selected the total stem part of *Caralluma adscendens* var. *Attenuata* to extract chemical constituents by cold maceration method and evaluated *Invitro* antioxidant activity by specified assay methods in the first step. The results obtained encouraged the researcher for further evaluation of *in vivo* antioxidant and hepatoprotective activities. Research methodology and the results obtained were described in the experimental section.

MATERIALS AND METHODS:

Materials

Collection of plant material

The whole stem part of *Caralluma adscendens* var. *attenuata* was collected from a rocky terrain near Nambulapulakunta village, Kadiri (Town), Anantapuramu district, Andhra Pradesh, India. Voucher specimens were deposited in S.K. University Herbarium (SKU) (Acc. No. SKU 51293).

Chemicals

All chemicals used for biochemical assays were of analytical grade and procured from Sigma Aldrich (Merck, Bangalore). Double distilled water was used as solvent in preparation of reagents and solutions.

Ethical Approval

The institutional Animal Ethics Committee (878/ac/05/CPCSEA/004/2017) has approved the experimental protocol to carry out the research at Department of Pharmacology, Raghavendra Institute of Pharmaceutical Education and Research, Anantapuramu, Andhra Pradesh, India.

Animals

Wistar Albino rats of both sexes (200-250 grams body weight) were purchased from Venkateshwara laboratories, Bengaluru for this study. They were housed at temperature (22±1°C), relative humidity (55±5% RH) and 12 h light/dark cycles. Animals were allowed to have free rat pellet diet and water was given *ad libitum* throughout the experiment.

METHODS

Preparation of extracts

The dried and powdered whole stem part of *Caralluma adscendens* var. *Attenuata* [5] was passed through a sieve no.22 and each kilogram of powder was extracted successively by cold maceration⁶ with 2.5 litres of methanol. The extracts were concentrated to dryness under reduced pressure using rotary vacuum evaporator and used for further investigation.

Phytochemical screening

Phytochemical screening of methanolic stem extract of *Caralluma adscendens* var. *attenuata* was carried out by chemical tests for identification of phenolics, flavonoids, tannins, terpenes alkaloids, glycosides etc [7].

In vitro Antioxidant activity

Determination of total phenolic content by Folin-Ciocalteu's method

The concentration of phenolics present in the methanolic extract was determined using Folin-Ciocalteu's reagent spectrophotometrically. 1mg/ml methanolic extract solution was used in the analysis. To the 0.5 ml of extract, 2.5 ml of 7.5% sodium bicarbonate solution and 2.5 ml of 10% Folin-ciocalteu's reagent were added. Blank was prepared without addition of plant extract. These were incubated at 45°C for 45 min. The absorbance of these solutions was determined at 710 nm spectrophotometrically. The same protocol was repeated thrice for samples and standard Gallic acid. The mean value of absorbance was calculated.

Standard curve was plotted for Gallic acid (Standard curve $y = 7.023x - 0.0182$, $r^2 = 0.999$). The content of phenolics in the extract was expressed in terms of Gallic acid equivalent (mg of GAE/g of extract). The concentration of total phenols was calculated with reference to Gallic acid as mg of GAE/g of extract [8].

Determination of flavonoid content by UV spectrophotometric method

The content of flavonoids present in methanolic extract was determined spectrophotometrically. 1mg/ml methanolic extract solution was used in the analysis. To 1 ml of 1mg/ml sample solution 0.5 ml of 2% ethanolic aluminium chloride solution was added. Blank solution was also prepared in the same manner by omitting the addition of plant extract. Then the absorbance was measured at 420 nm using UV spectrophotometer. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. Rutin was taken as standard solution and the same protocol was applied. (Standard curve $y = 16.212x - 0.0582$, $r^2 = 0.999$). The flavonoid content present in the extracts was considered as rutin equivalent (mg of RUE/g of extract) [8].

Reductive Ability

The Reductive ability of the plant extracts was determined according to Oyaizu method [9]. Methanolic extract of *Caralluma adscendens* var. *attenuata* was dissolved in 1ml of distilled water to this 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of Potassium ferric cyanide [$K_3Fe(CN)_6$] (1%) were added. The mixture was then centrifuged at 3000 rpm for 10 min. Then 2.5 ml of the upper layer of the solution was mixed with 2.5 ml of distilled water and 0.5ml of $FeCl_3$ (0.1%). Absorbance was measured at 550nm. Butylated Hydroxyl Toluene (BHT) was used as reference compound. All the analysis was performed in triplicate. Reducing ability (%) was calculated according to formula (1).

$$= \frac{(V_0 - V_1)}{V_0} \times 100$$

V_0 = absorbance of control and the
 V_1 = absorbance of the sample.

Free Radical Scavenging Activity (FRSA) using hydrogen peroxide

The hydrogen peroxide FRSA¹⁰ of the methanolic extract was done as suggested by Czochra and Widwonsk. According to this method to 1.0 ml of methanolic sample (100 μ g / ml), 2 ml of hydrogen peroxide (30 %) and 2.4 ml of 0.1 M phosphate buffer (pH 7.4) were added. The resulting solution

was kept for 10 min. Absorbance was recorded at 230 nm. All readings were repeated thrice. Blank was prepared without adding hydrogen peroxide and control was prepared without a sample. Ascorbic acid was used as a standard compound. Free radical scavenging activity of hydrogen peroxide (%) was calculated as per formula (1).

Acute toxicity studies

According to OECD NO: 420¹¹ guidelines given by the Organization for Economic Cooperation and Development acute oral toxicity studies were performed for methanolic extracts of *Caralluma adscendens* var. *Attenuata*. Wistar albino rats of both sexes weighing 200 to 250 g were used for the study. These animals were subjected for overnight fasting and divided into four groups, as each group consists of three animals. 5mg/kg bw of methanolic extract of *Caralluma adscendens* var. *Attenuata* was orally administered for the animals of all groups. These were observed for 1 hr and consequently 3 hrs for signs of toxicity like skin rashes, lacrimation, sneezing, hair fall and mortality. Since no mortality was observed at this dose, 50 mg/kg, 300 mg/kg and 2000 mg/kg of extracts was given to all the groups again. These animals were observed up to 14 days after oral administration of the extract to notify any toxicity or delayed mortality. 2000 mg/kg bw was recognized as non- toxic dose for oral administration according to OECD guidelines for single herbal extract.

Selection of dose

1/10th of the non-toxic maximum dose was selected as low dose and 1/5th of non-toxic maximum dose was selected as high dose to carryout *in vivo* antioxidant and hepatoprotective studies.

Experimental design for *In vivo* study

To study *In vivo* antioxidant and hepatoprotective activities, 30 Wistar albino rats of both sexes were randomly divided into 5 groups as each group consisting of 6 animals and the experiment was designed to carry out 21 days with single dose administration (every 24 hours) by the oral route.

Group 1: Normal control rats were received 2 ml of 2% gum acacia (0.1 g/200 g bw) for 21 days.

Group 2: Negative control rats received 2g/kg/day ethanol (40%) for a period of 21 days.

Group 3: Received 1ml of 2% acacia suspension containing 50 mg/kg bw of silymarin as standard¹² along with 2g/kg/day ethanol (40%) as daily dose.

Group 4: Received a solution containing 2g/kg/day ethanol (40%) and 200 mg/kg bw of methanolic

extract of *Caralluma adscendens* var. *Attenuata* for 21 days.

Group 5: Received a solution containing 2g/kg/day ethanol (40%) and 400 mg/kg bw of methanolic extract of *Caralluma adscendens* var. *Attenuata* for 21 days.

On 22nd day blood samples were collected by retro-orbital plexus then transferred into a sterilized centrifuge tube, allowed to clot and serum was separated at 2500 rpm about 15 min to evaluate various biochemical parameters for liver function study by estimation of Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP) according to King (1965) method [13], serum bilirubin levels as reported by Malloy et al., (1937) [12] respectively using the diagnostic kits purchased from Sigma Aldrich (Bengaluru, India).

To study histopathology [14-16] the test animals were sacrificed, liver tissues were removed by cervical dislocation and then fixed in 10% buffered formalin which was dehydrated with alcohol. Liver tissues were then cleaned and embedded in paraffin, cut into 3–5 μ m sections, stained with the haematoxylin-eosin dye and finally observed under a photomicroscope to investigate cell necrosis, fatty changes or inflammation of lymphocytes etc[17]

To estimate antioxidant activity, after excision of liver tissue for histopathological studies the remaining liver of each animal was rinsed in ice-cold normal saline, followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10% w/v of homogenate was prepared; few ml of trichloro acetic acid (TCA) was added to precipitate proteins, filtered then centrifuged at 15000 rpm for 15 min at 4°C. The supernatant thus obtained was used for estimation of Superoxide dismutase (SOD) ¹⁸, Catalase (CAT), Reduced glutathione (GSH) levels according to the standard methods specified by Mishra & Fridovich (1971), Hugo E. Aebi (1979), Moran M.S et. al (1979) respectively [19].

Statistical analysis

The results obtained for the above mentioned biochemical parameters were applied for statistical analyses using Graph Pad prism 7.0 version software. The data was expressed as mean with Standard deviation. Student's paired *t* test was used to determine the significance of the study so as to observe the *P*-value is less than 0.05.²⁰

RESULTS:

The phytochemical screening of the methanolic extract had revealed the presence of phenolics, flavonoids, tannins, terpenes alkaloids, glycosides etc. as mentioned in Table 1.

Table 1: Phytochemical analysis of methanolic extract of *Caralluma adscendens* var. *attenuata*

Phytoconstituents	Methanolic extract of <i>Caralluma adscendens</i> var. <i>attenuata</i>
Phenolics	+ ve
Flavonoids	+ ve
Tannins	+ ve
Terpenes	+ ve
Alkaloids	+ ve
Glycosides	+ ve
Vitamins (A & D)	+ ve
Calcium, Magnesium, Zinc	+ ve

Invitro antioxidant activity

The results obtained for the specified assays were represented in Table 2 indicating a highly significant amount of antioxidants were present in the methanolic stem extract.

Table 2: Results of Invitro antioxidant activity tests

Name of the Assay	Content present in methanolic extract *
Total phenolic content	22.5 $\pm$ 0.09 mg GAE/g
Total flavonoid content	6.2 $\pm$ 0.05 mg RUE/g.
FRSA	88.41 % $\pm$ 0.230
Reductive ability	96.13 % $\pm$ 0.150

*Mean values (n = 3) with significant difference at *P* < 0.05

In vivo antioxidant activity

Various biochemical parameters related to *Invivo* antioxidant and hepatoprotective activities were studied. The results obtained were represented in Table 3 & 4.

Table 3: Effects of methanolic extract of *Caralluma adscendens* var. *Attenuata* on serum biochemical parameters against ethanol induced liver injury

Group	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Total bilirubin (µg/dL)
Normal range	45.7 – 80.8	17.5 – 30.2	56.8 – 128	0.2 – 0.55
Group I (Normal)	50.1 ± 2.53	25.6 ± 3.56	55.8 ± 3.57	0.48 ± 1.52
Group II (Ethanol)	123.8 ± 1.59*	53.2 ± 2.14*	140.5 ± 4.12*	2.82 ± 1.26*
Group III (standard)	46.7 ± 3.54	35.4 ± 2.56	50.7 ± 3.56	0.59 ± 1.47
Group IV (Low dose)	61.4 ± 3.99	42.3 ± 3.09	74.9 ± 3.01	0.46 ± 1.21
Group V (high dose)	50.3 ± 2.61*	40.7 ± 2.15*	53.4 ± 4.22*	0.21 ± 1.42*

Values are mean ± SD., n = 6 animals in each group. Statistical significance is indicated by asterisks. **p* < 0.05 against ethanol induced liver injury

Table 4: Effects of methanolic extract of *Caralluma adscendens* var. *Attenuata* on antioxidant enzymes

Groups	Super oxide dismutase (U/mg)	Catalase (µM/min/mg)	Reduced glutathione (µg/mg)
Group I (Normal)	48.4 ± 1.41	39.2 ± 1.21	35.8 ± 1.21
Group II (Ethanol)	23.1 ± 1.01	28.5 ± 2.82	18.2 ± 1.58
Group III (standard)	28.5 ± 0.21	33.8 ± 2.10	22.1 ± 1.41
Group IV (Low dose)	15.2 ± 0.31	15.9 ± 0.92	15.6 ± 1.73
Group V (high dose)	20.5 ± 1.58	22.6 ± 2.12	25.3 ± 2.12

Values are mean ± SD., n = 6 animals in each group. Statistical significance is indicated by asterisks. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001 vs. ethanol induced liver injury.

DISCUSSION:

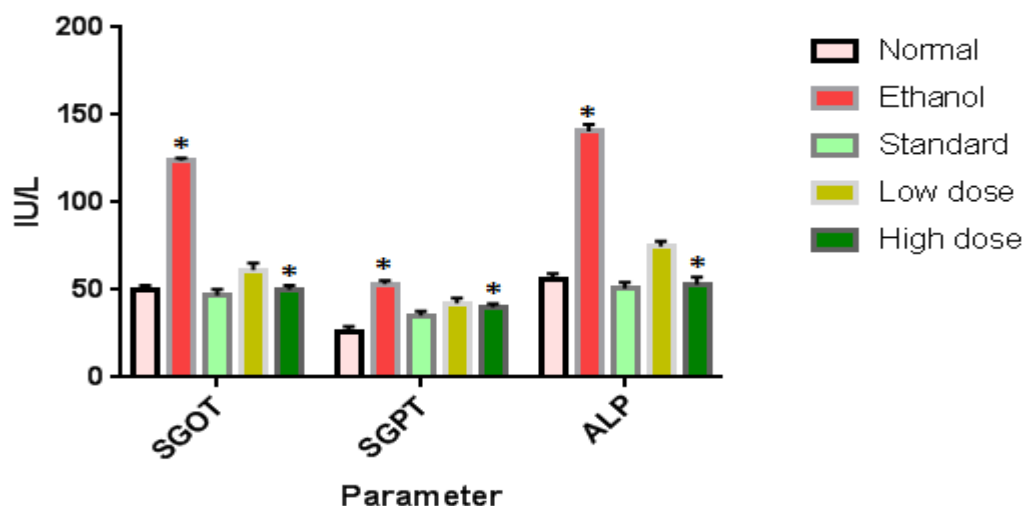
Flavonoids and total phenolic compounds serve as good antioxidants. These are abundantly distributed in medicinal herbs and also have better hepatoprotective property. In the present study the positive results obtained for phyto chemical investigations and *In vitro* antioxidant studies of methanolic extract of *Caralluma adscendens* var. *Attenuata* had become an ideal proof to continue the research to evaluate *Invivo* antioxidant and hepatoprotective activities.

SGOT is an enzyme present in cardiac and hepatic tissues. It involves in metabolism of aminoacids, aspartate and alpha ketoglutarate to oxaloacetate and glutamate respectively. So SGOT is released into blood when abnormal liver function is associated with aminoacid metabolism which indicates liver damage or inflammation. Location of SGPT is same as that of SGOT, it catalyses the amino group transfer from alanine to alpha ketoglutarate consequently increases the serum levels of pyruvate and glutamate. Liver damage or myocardial infarction significantly

increases serum SGPT levels. ALP is an enzyme produced and regularized on the outer membrane of bile ducts. If bile duct is damaged by hepatic cholestasis and/ infiltrative diseases generally increases serum ALP levels. Total bilirubin is the sum of both unconjugated and conjugated bilirubin. Unconjugated bilirubin is a breakdown product of heme. In normal functioning of the liver both forms of bilirubin would be absorbed by the liver, whereas addition of high concentration of some of the drugs and free fatty acids can cause poor absorption ultimately which may appear in blood. Elevated levels of bilirubin is the significance of liver disorders or injury.

In the present research increased levels of SGOT, SGPT, ALP and bilirubin were observed in ethanol treated Group II when compared with Standard silymarin Group III, Group IV & V. Retention to the normal range of the above biochemical parameters was observed in Group V treated with 400 mg/kg bw of methanolic extract of *Caralluma adscendens* var. *Attenuata* was shown in Fig. 1.

A



B

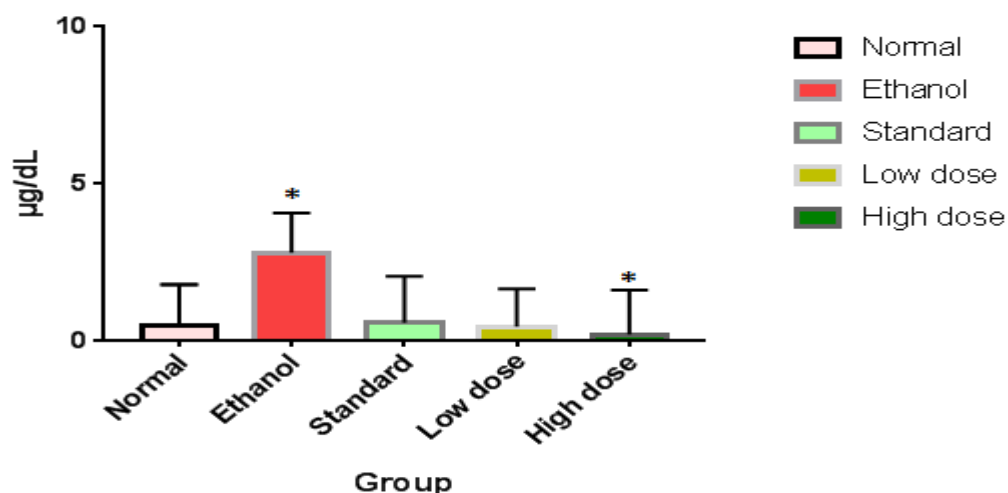


Figure 1: Effects of methanolic extract of *Caralluma adscendens* var. *Attenuata* on serum biochemical parameters against ethanol induced liver injury. (A) Representation of SGOT, SGPT, ALP. (B) Representation of Total bilirubin. Group I: Normal, Group II: Ethanol, Group III: Standard, Group IV: Low dose, Group V: High dose.

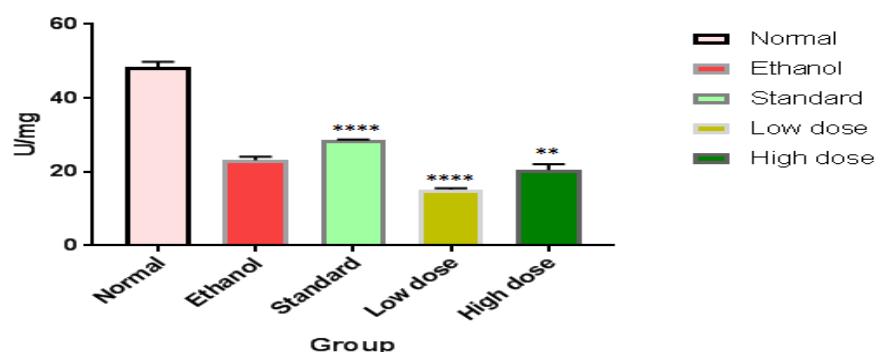
Values are mean $\pm$ SD., n = 6 animals in each group. Statistical significance is indicated by asterisks. * $p < 0.05$ against ethanol induced liver injury.

Superoxide (O_2^-) is a by-product of oxygen metabolism and it would be converted to normal oxygen (O_2) by dismutation (partitioning) in catalyses of SOD enzyme. If $O_2^- \rightarrow O_2$ conversion is not regulated properly it may cause cell damage especially hepatic and cardiac cells. Since SOD is an specific antioxidant defence enzyme normal ranges of SOD should be present in healthy living beings. Catalase is an enzyme that facilitates the conversion of $H_2O_2 \rightarrow H_2O + (O)$ and also protects the cell from

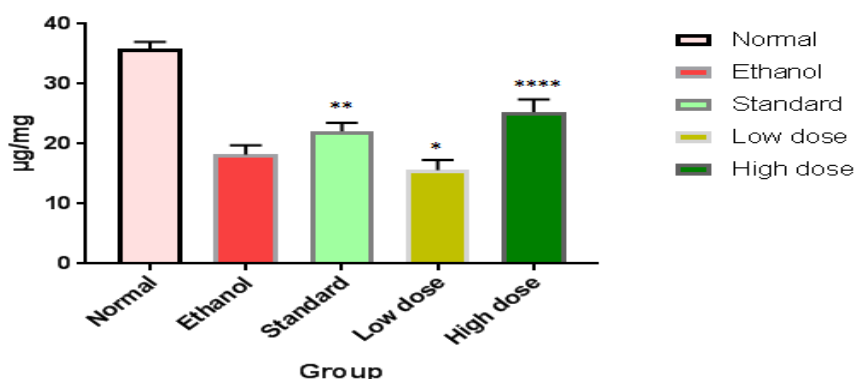
oxidative degeneration by ROS. GSH is a non-biological antioxidant. It protects cells against free radicals, peroxides and other toxic compounds. It plays a vital role in coordinating the antioxidant defence mechanism of system. Normal range of the above biochemical parameters shall be generally seen in healthy living beings. Low levels of these parameters indicate abrupted antioxidant defence mechanism, hepatic inflammation and necrosis.

The antioxidant marker enzymes were significantly decreased in ethanol treated Group II when compared with standard silymarin Group III, Group IV & V. Significant restoration of these enzymes was observed in Group V than Group IV was shown in Fig. 2.

A



B



C

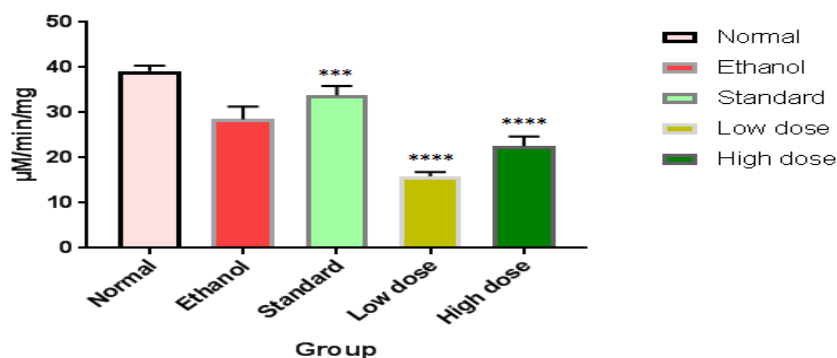


Figure 2: Effects of methanolic extract of *Caralluma adscendens* var. *Attenuata* on antioxidant enzymes against ethanol induced liver injury. (A) Representation of SOD (B) Representation of GSH (C) Representation of CAT. Group I: Normal, Group II: Ethanol, Group III: Standard, Group IV: Low dose, Group V: High dose.

Values are mean $\pm$ SD., n = 6 animals in each group. Statistical significance is indicated by asterisks. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. ethanol induced liver injury.

Normal histology of hepatic cells include well distributed hepatocyte clusters appear in poly or hexagonal shape along with well-defined intact nucleus, closely arranged sinusoid spaces, prominent hepatic veins and without any clump of monocytes.

In the present study liver sections of normal group Fig. 3A treated animals has shown idealistic histology. Where as in ethanol treated group has shown damage in the central vein, dilatation of sinusoidal space, indefinite cell membrane, blobbing of hepatocytes, presence of fatty lobules and clusters

of monocytes indicating hepatic tissue injury and mild necrosis Fig. 3B. Silymarin treated group has shown repaired hepatocytes and closure sinusoidal spaces Fig. 3C. Group IV treated animals has shown moderately repaired hepatic tissue with a few monocytes and normalized hepatic veins Fig. 3D. Histology of group V has shown nearly normal hepatic architecture illustrating recovery of hepatocytes without infiltration of RBC in central vein, with prominent nuclei and no lipid depositions were observed Fig. 3E.

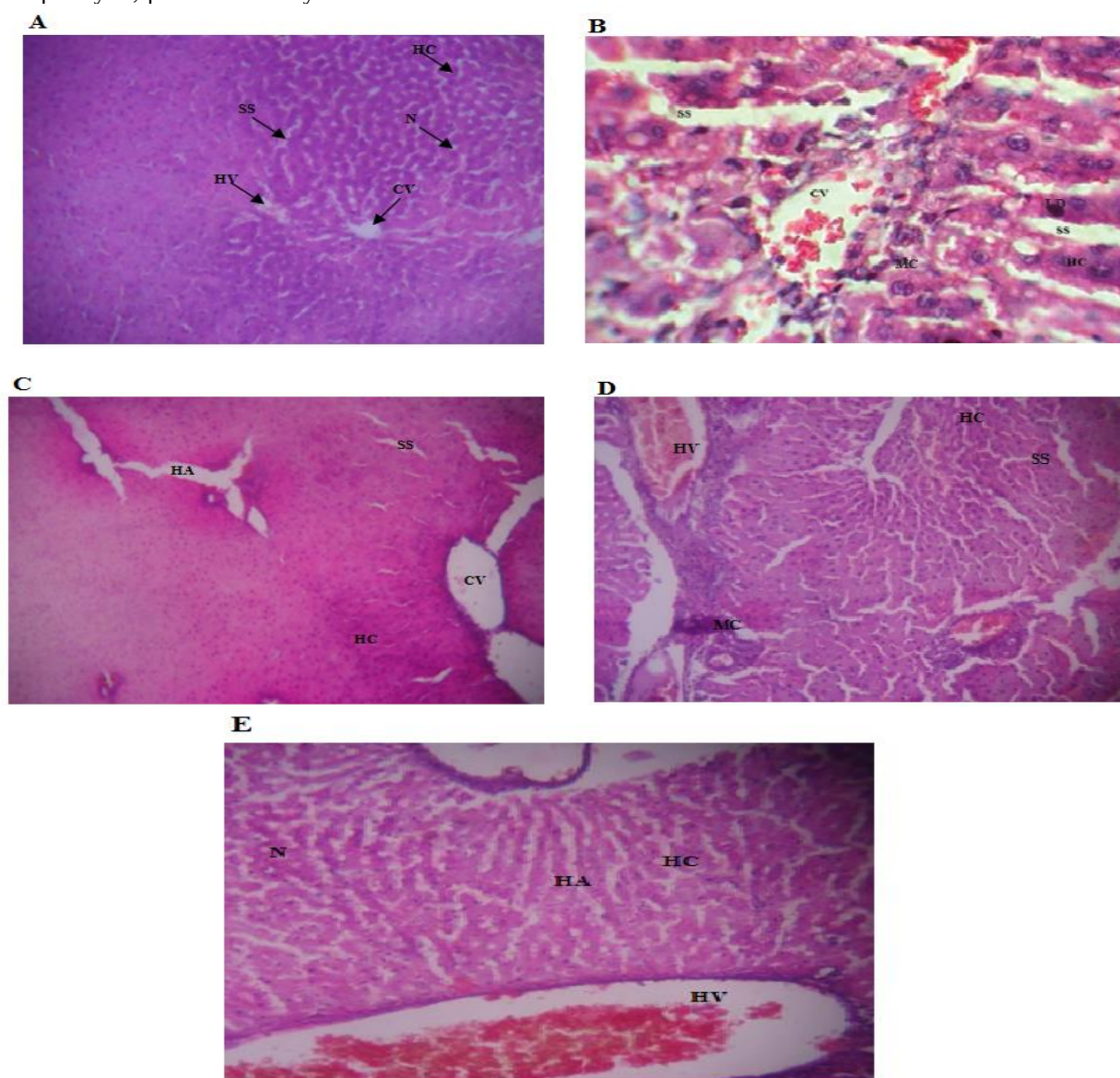


Figure 3: Histological micrograph of rat liver sections against ethanol induced hepatotoxicity (100 X). (A) Group I: Normal (B) Group II: Ethanol (40%) (C) Group III: Standard (50 mg/kg bw) (D) Group IV: Low dose (200 mg/kg bw) (E) Group V: High dose (400 mg/kg bw). HA: Hepatic artery; CV: Central vein; HC: Hepatocytes; MC: Monocytes; N: Nuclei; SS: Sinusoidal spaces; LD: Lipid deposits.

CONCLUSION:

The above findings of the present research revealed that *Caralluma adscendens* var. *attenuata* has significant antioxidant activity and hepatoprotective potential against ethanol induced liver toxicity. Hence we advocate the researchers to investigate the plant extract for further half-life prediction, toxicological studies and herbal formulation etc.

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